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13. ABSTRACT *(Maximum 200 words)*

We have found that breast cancer cell expression of the transferrin receptor (TfR) and cellular ability to proliferate in response to transferrin (Tf) are properties which correlate with the ability of mammary cancer cells to metastasize. The current grant focuses attention on different mechanisms for elevating or depressing breast cancer cell TfR expression and to assess any changes in metastatic capability caused by these alterations. The current objectives are: (1) to raise tumor cell TfR levels by transfection with a sense TfR plasmid construct; (2) to decrease breast cancer cell TfR levels by transfection with an antisense TfR plasmid construct; (3) to select high or low TfR expressors by the use of various growth selection or FACS techniques. We have been able to increase breast cancer cell metastatic ability by the use of a Tf growth selection process. Transfection of tumor cells with a sense TfR construct has resulted in cells with markedly higher TfR expression. However, increased TfR expression does not necessarily cause an increased proliferative response to Tf. Transfection with a TfR antisense construct have not yet resulted in a depression of TfR levels. Studies which define cellular properties that link increased TfR expression to increased response to Tf are indicated.

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INTRODUCTION

A. Background

Understanding the mechanisms of tumor cell metastasis is of paramount importance in attempting to resolve the cancer problem. Metastasis of breast cancer cells from the primary site to distant locations is the major cause of death from this disease (1). Specific factors present at the preferred site for metastasis and the ability of tumor cells to respond or interact with those factors is thought to be necessary for the successful formation of metastatic lesions (2).

Previous studies by us explored the possibility that tumor cell metastasis to a certain target organ was in part enhanced by the ability of the tumor cells to respond to growth factors encountered in the target organ environment. Cells of high and low lung-metastasizing capability from the rat 13762NF mammary adenocarcinoma were examined for their ability to proliferate in response towards media conditioned by lung fragments. A response was seen for the high lung-metastasizing cells only. The major lung-derived lung metastasizing tumor cell mitogen was purified from the conditioned media and was found to be the iron transport protein transferrin (Tf;3-5). Subsequent studies showed a correlation between tumor cell response to transferrin and metastatic capability in 5 of 6 animal and human tumor model systems (6,7).

The transferrin receptor (TfR), a M_r ~180,000 homodimeric integral membrane glycoprotein (8), binds two iron saturated Tf molecules and is responsible for the delivery of iron into cells either through internalization of Fe-Tf (9) or by activation of a plasma membrane associated NADH dependent oxidoreductase which mediates the trans-plasma membrane transport of iron from Tf (10). Rapidly dividing cells, including a variety of tumor cells (8,11,12), usually express high levels of TfR. Other investigators have shown that tumor cell expression of TfR, as determined by histochemical analysis, has been shown to correlate with tumor grade or stage and/or progression and metastasis in human breast carcinomas (13), human bladder transitional cell carcinomas (14), and in human malignant melanoma (15). High levels of TfR have been observed in a metastatic lesion of a maxillary neoplasm, but not in the parental tumor (16). Transferrin receptor expression was seen to be increased in a human melanoma line selected for metastatic capability in nude mice, when compared to the poorly metastatic parental population (17). Transferrin has been shown to be the major mitogen in bone marrow for bone marrow metastasizing human prostatic carcinoma cells (18), and proliferative response to Tf has been shown to be associated with malignant progression in a series of murine B16 melanoma sublines (19).

Transferrin's proliferative effect on cells is thought to be due primarily to its ability to transport iron into the cell, thus maintaining the activity of key enzymes required for proliferation. One major site of iron need in rapidly dividing cells is the enzyme ribonucleotide reductase (8). However, some studies have suggested that iron transport alone cannot explain transferrin's growth stimulating activity (20-22). Also, iron delivered by Tf has been shown to mediate processes that soluble iron can not (23), and stimulation of the TfR through transferrin-independent means has been shown to induce a number of activities in T cells (24).

The observation of a cellular property associated with and perhaps thought to be responsible for metastatic activity has frequently led metastasis researchers to pursue *in vitro* selection techniques whereby tumor cells possessing high or low levels of a metastasis-associated marker or activity could be isolated. The proposed influence on metastatic behavior that the selected property played could then be determined by assessing the metastatic capability of the selected cells *in vivo* (25-29). These types of experiments have resulted in the identification of a number of tumor cell phenotypes thought to be associated with the ability to form metastases (25-29).

The transfection of low or high metastaic tumor cells with sense or antisense genes encoding factors thought to affect metastatic behavior has also been a frequently performed procedure. Thus, the molecular approach to elevating or depressing the expression of a given tumor cell protein could be

performed to determine the affect of that expression on metastatic capacity. A number of studies have been performed whereby the sense expression of a protein has been shown to influence metastatic capability (30-32). Likewise, similar types of studies have demonstrated that the depletion of a factor by antisense expression of its message has altered the invasive capability of malignant cells (33).

Based on our previous obsevations, we thought that the determination of a possible involvement of TfR in breast cancer metastasis could be explored by breast cancer cell selection or transfection techniques. The transfection of cells with the sense gene for TfR has been accomplished by others (34.35). The bulk of these studies were performed using normal cells, the purpose to gain basic information on the functioning of TfR. One report has been published whereby the sense or antisense TfR gene has been transfected into tumor cells (35). However, no conclusions were made about the alteration of any tumor cell behaviors caused by the transfection process.

B. THE PURPOSES

The major purposes are:

B-1. To transfect low metastatic mammary tumor cells with the sense gene for the transferrin receptor and to determine the affect of increased expression on tumor cell proliferative response to Tf and on metastaic capability.

We have found that tumor cell TfR expression and/or proliferative response to Tf correlates (in many cases) with metastatic capability. Since a major body of work has been accomplished by others using molecular biology techniques and transfection technology to produce and examine the affect of increased expression of a protein on cell function, the use of these types of procedures was a natural consideration in the study of TfR expression on tumor cell behavior.

B-2. To transfect highly metastatic cells with the antisense gene for the transferrin receptor and to determine the affect of decreased TfR expression on tumor cell proliferative response to Tf and on metastatic capability.
The expression of many cellular factors may be responsible for high metastatic capability. Increasing the expression of one of these in a low metastatic cell hypothetically deficient in a number of responsible factors, would therefore have no effect. However, if a highly metastatic cell is depleted of one factor necessary for metastatic function, then the chances of observing a decrease in metastatic behavior would be greater. Thus, the transfection of cells with a plasmid which would produce constitutive expression of an antisense TfR message is a more logical choice of experiments than that outlined in B-1.

B-3 To isolate high or low TfR expressing tumor cells from a mixed population by using selection or sorting techniques. To determine the metastatic capability of the selected cells.
As mentioned in the background section, many in vitro selection techniques have been used to obtain populations of tumor cells which are more or less metastatic than the parental population. As these types of experiments can also make strong statements about cellular requirements for metastatic behavior, they are a logical choice. Initially, cells will be selected for enhanced response to Tf using an *in vitro* selection process, and cells expressing high or low levels of TfR will be sorted using FACS.

BODY:

B1. Transfection of mammary tumor cells with the sense gene for TfR.
The following tumor cell lines were used:

Cell Line:	**Metastatic Capability:**	**TfR Expression:**	**Growth in Response to Tf:**
Human A375p Melanoma (37)	Low	Low	Low
Human MDA 468 Mammary Adenocarcinoma (38)	Low	Low	Low
Human MDA 231 Mammary Adenocarcinoma (38)	Good	Very Low	Good

A sense TfR encoding construct was made by ligating the TfR coding region from pcDTR1 into the MCS of pcDNA1Neo. The resulting plasmid was named pcDNA1/Neo/TfR (Figure 1). Vector only transfected cells were transfected with the pcDNA1Neo plasmid only. All lines were transfected using Lipofectamine and were selected using 300 µg/ml G418, and maintained in media containing 400 µg/ml G418.

B1:A375p cells

A375p: This line was initially transfected to determine the usefulness of the TfR construct. The uncloned TfR (A375p/TfR) transfected population possessed 3-4 more TfR than did the vector transfected controls (A375p/Neo), based on immunofluorescent studies, and on measurement of TfR by affinity isolation (Fig 2). However, FACS analysis revealed only a 1.5 fold increase in TfR in the A375p/TfR cells (Fig 3). Theses cells, when examined for spontaneous or experimental metastatic capability in nude mice, produced no more metastases than did the Neo controls (Table 1). To further increase TfR expression in the TfR line, the top 2% TfR expressors were isolated by FACS. These cells were re-cultured, and when re-analyzed displayed a similar TfR expression as did the unsorted population (Figure 4).

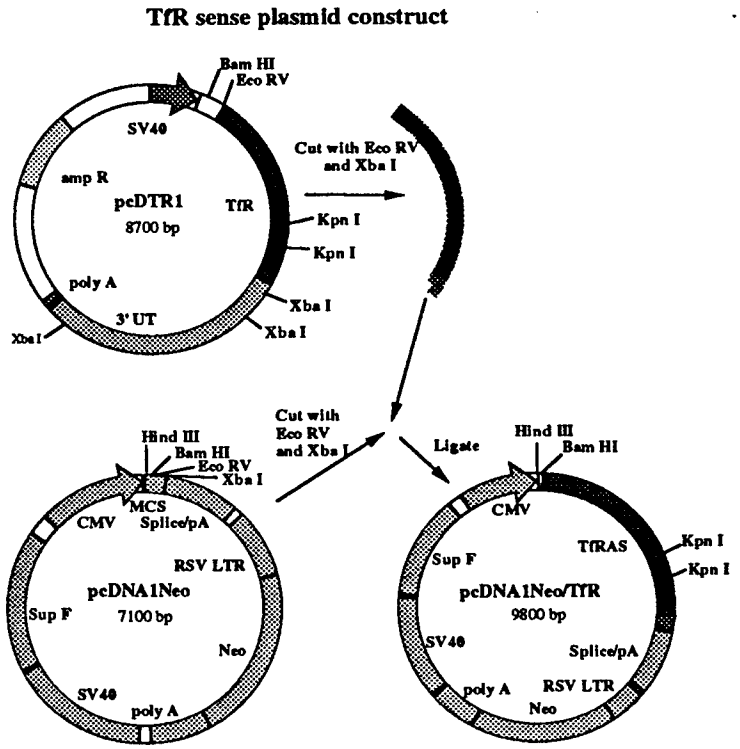

Figure 1. Construction of the eucaryotic expression vector containing the sense cDNA for the human transferrin receptor. The coding region was cut out of pcDTR1 using ECO RV and Xba I and was ligated into the MCS of pcDNA1Neo, which had been cut with the same two enzymes.

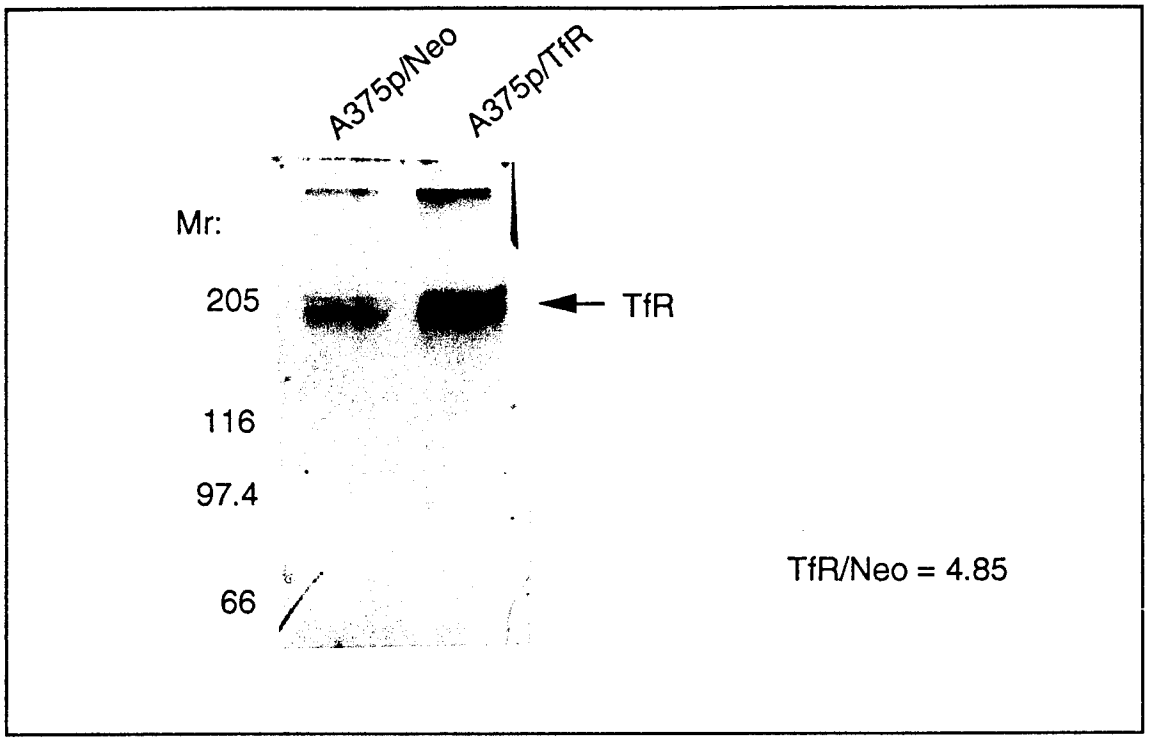

Figure 2. Results of the affinity isolation of biotinylated TfR from A375p/Neo and A375p/TfR cells. The procedure for the affinity isolation technique is more completely described in the appendix. Cells are surface biotinylated at 4°C, lysed, and equal amounts of cell lysate protein are exposed to an excess of Tf-agarose. The agarose is washed extensively and bound material is removed by SDS-PAGE treatment solution. Released proteins are separated by SDS-PAGE and blotted onto PVDF. The blot is incubated with streptavivdin-HRP and HRP bands are visualized by exposure to ECL HRP substrate followed by autoradiography.

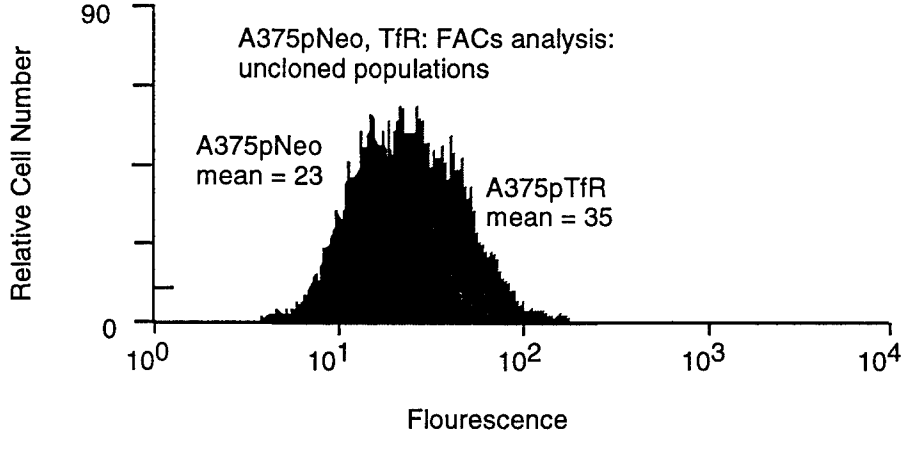

Figure 3. Initial analysis of TfR expression on A375p/Neo and A375p/TfR cells by FACS. Cells were removed form plates by brief exposure to trypsin, and were incubated for 2h at 4°C with a phycoerythrin conjugated monoclonal anti-human TfR. Cells were washed and analyzed for fluorescence.

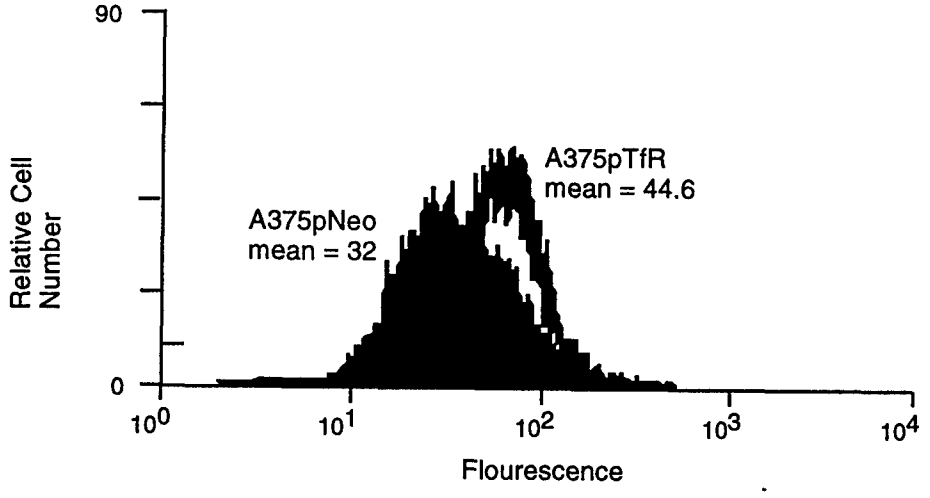

Figure 4. Analysis of TfR expression on A375p/Neo and A375p/TfR cells by FACS. The top 2 % of TfR expressing cells from the A375p/TfR population shown in figure 3 were re-cultured, expanded and re-analyzed for TfR expression.

Table 1: Results of spontaneous metastases assays:

A375pNeo:

Mouse#	Primary Tumor Volume (cm^3)	Metastases
1	2.5	0
2	2.4	0
3	2.0	0
4	1.5	0
5	1.0	0
6	1.0	0

A375pTfR:

1	2.0	0
2	1.5	0
3	1.3	0
4	1.0	0
5	0.8	0
6	0.0	0

1 X 10^6 cells were injected in a 0.2 ml volume into the right flank of Metofane anesthetized male nude mice (age: 6 - 8 weeks). Eight weeks later, animals were sacrificed with an overdose of Metofane and were examined for the presence of metastatic lesions.

Table 2: Experimental Metastasis Results:

A375pNeo:

Mouse#	Metastases	Micrometastases:
1	0	0
2	0	0
3	0	
4	0	
5	0	
6	0	

A375pTfR:

1	0	0
2	0	0
3	0	
4	0	
5	0	
6	0	

1 X 10^6 cells in a 0.1 ml volume were injected into the lateral tail vein of male nude mice. Ten weeks later (first three mice) or six months later (second three mice), animals were sacrificed with an overdose of Metofane and were examined for the presence of metastatic lesions.

B1: MDA468 cells

MDA468: As with the A375p, the uncloned TfR (MDA468/TfR) transfected population possessed 3-4 more TfR than did the vector transfected controls (MDA468/Neo), based on immunofluorescent studies, and on measurement of TfR by affinity isolation (Fig 5). However, FACS analysis revealed only a 1.5 fold increase in TfR in the MDA468/TfR cells (Fig 6). Work on these cells has been tabled, however, plans are to sort out high expressors using FACS. There appears to be a population of high TfR expressors in the TfR population. as the FACS analysis shows a "tail" on the right side.

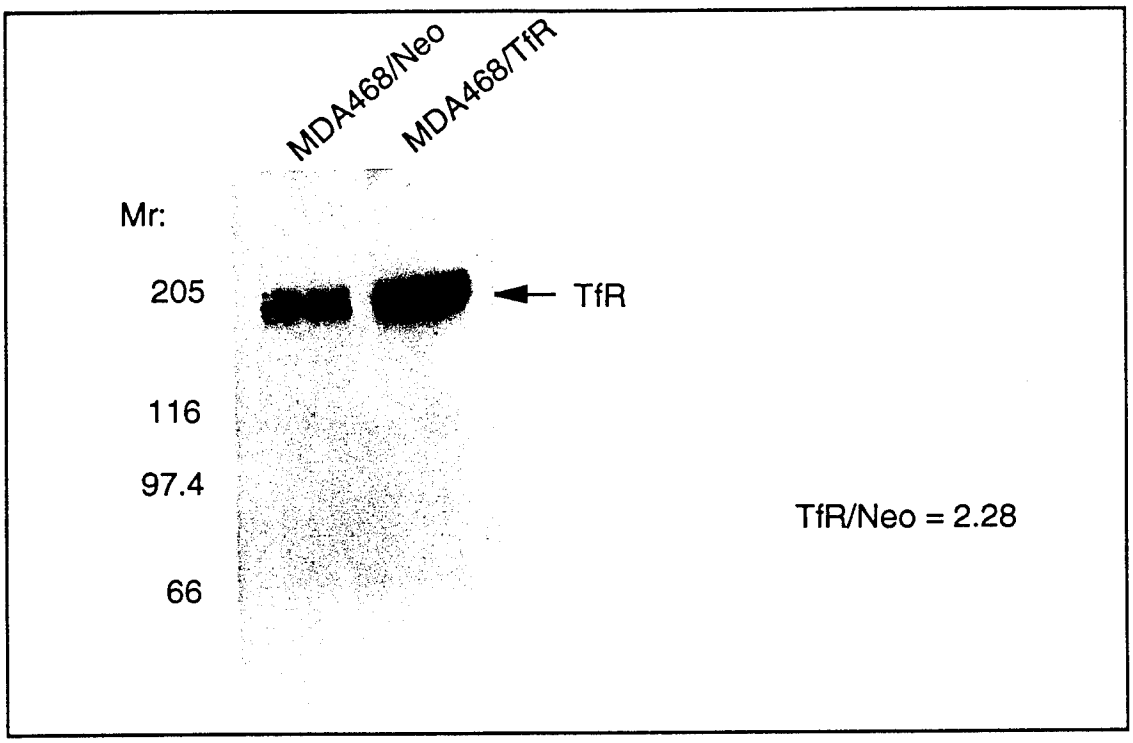

Figure 5. Results of affinity isolation of TfR from MDA468/Neo and MDA478/TfR cells. The method used was identical to that stated in the legend for figure 2.

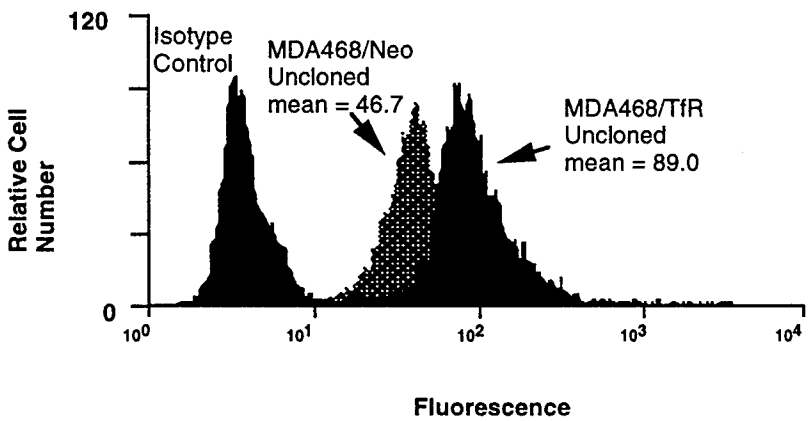

Figure 6. Initial FACS analysis of TfR expression on uncloned, unsorted MDA468/Neo and MDA478/TfR cells. The staining procedure is identical to that stated in the legend for figure 3.

B1:MDA231 cells

MDA231: This intermediately metastatic line (38), this line was used for transfection studies because, in our hands, it expresses extremely low levels of TfR. When analyzed for TfR expression by FACS, the TfR stained cells often produce a profile identical to the isotype control. So, the line came to be an excellent indicator of the ability of our plasmid to increase TfR expression, since background expression is almost zero. By immunofluorescent analysis, 1-2% of the uncloned MDA231/TfR population displayed markedly increased TfR expression. The cells were cloned, and 3/20 clones displaying increased TfR expression were obtained. The highest expressor was expanded and re-analyzed. These cells exhibited a much greater, but heterogenous, expression of TfR than did the Neo controls (Figure 7). Synchronization of the cell into the G1/S phase by administration of aphidocolin did not affect the heterogeneity of the expression (data not shown). The top 2% of the TfR expressors were isolated by FACS sorting, and re-cultured. The sorted population was then identically re-sorted. This process has been repeated four times, with the cells from the fourth sort displaying a slightly higher TfR level than those from the first sort (Figure 8). Analysis of TfR by affinity isolation also revealed a marked increase in TfR in the TfR transfected, sorted cells (Figure 9). These cells have recently been injected into the mammary fat pad of nude mice, to assess their spontaneous metastatic capability.

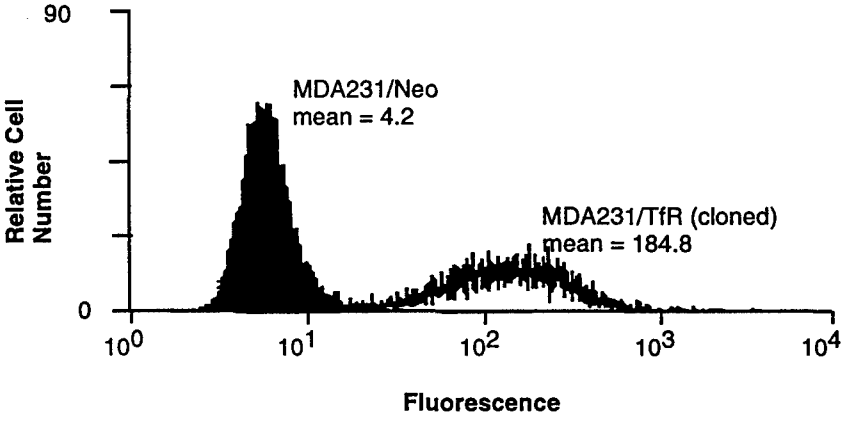

Figure 7. Initial FACS analysis of TfR expression on MDA231/Neo and cloned MDA231/TfR cells. The staining procedure is identical to that stated in the legend for figure 3.

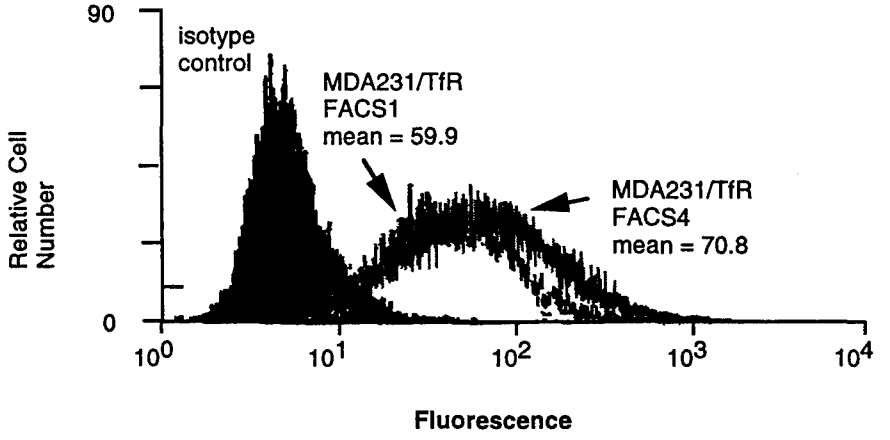

Figure 8. Results of FACS analysis of TfR expression on MDA231/Neo cells and MDA231/TfR cells obtained from the first and fourth sequential sorts for high TfR expressors. The staining procedure is identical to that stated in the legend for figure 3.

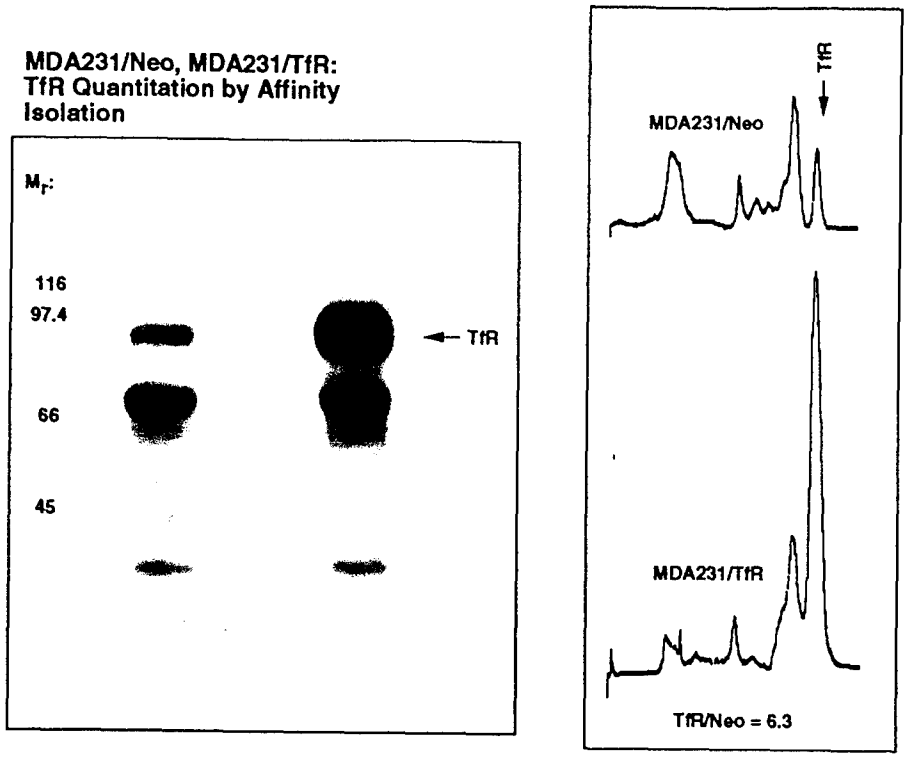

Figure 9. Results of affinity isolation of TfR from MDA231/Neo and MDA231/TfR (FACS sort 4) cells. The method used was identical to that stated in the legend for figure 2.

B2. Transfecting highly metastatic cells with the antisense gene for TfR.

The highly metastatic MCF7/LCC2 (39,40) cell line has been used for these experiments. In our hands, this line expresses very high levels of TfR (7). The construction of a full length antisense TfR construct was completed as diagrammed in figure 10. Detection of bacterial clones whose plasmid contained the TfR cDNA in an antisense orientation was accomplished by analysis of fragments produced by Bam HI, Kpn I digestion, wherein the antisense plasmid produces a produced a shorter Bam HI - Kpn I fragment. Transfection procedures were identical to those described above. In two separate transfection experiments, uncloned TfR antisense transfected MCF7/LCC2 (MCF7/LCC2/TfR^{AS}) populations displayed higher TfR expression than did Neo transfected controls. A FACS analysis of cells from the second experiment is shown in figure 11. We are currently attempting to determine why this is and are re-evaluating the make-up of the antisense plasmid. Attempts are also being made to construct another antisense plasmid, where the Bam HI - Kpn I TfR fragment from pcDTR1 will be ligated into pcDNA3, which possesses novel sites in its MCS region for those same two enzymes, but in a reverse orientation.

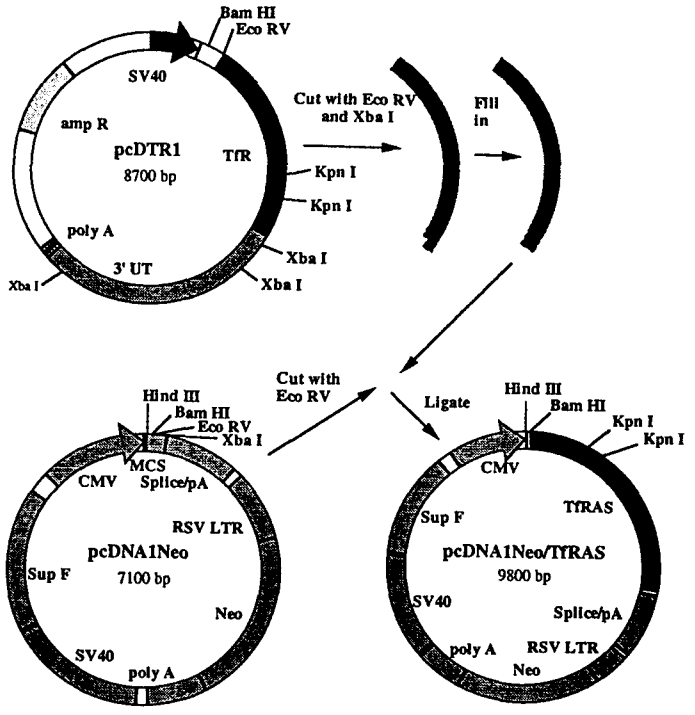

Figure 10. Construction of the eucaryotic expression vector containing the antisense cDNA for the human TfR. The cDNA for TfR was cut from pcDTR1 using Eco RV and Xba I. The fragment was blunt-ended and ligated into pcDNA1Neo which was cut with Eco RV. Plasmids containing cDNA of antisense orientation were verified by the presence of a smaller Bam HI- Kpn I fragment.

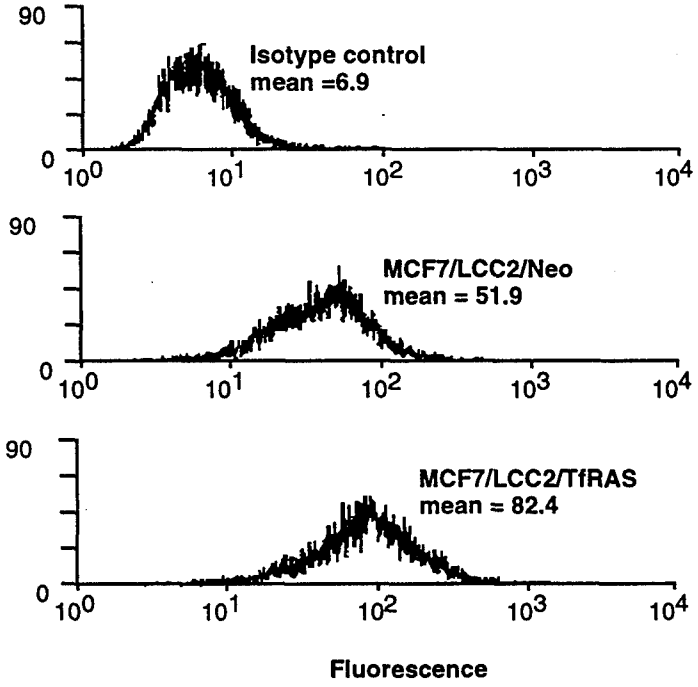

Figure 11. Results of FACS analysis of MCF7/LCC2/Neo cells and MCF7/LCC2/TfRAS cells. The procedure used is identical to that stated in figure 3 and shows a higher expression in the antisense transfected cells.

3. To isolate high or low TfR expressing tumor cells from a mixed population by using selection or sorting techniques. To determine the metastatic capability of the selected cells.

Using an *in vitro* growth selection process, a high TfR expressing, highly Tf-responsive cell line has been selected from the low metastatic, low Tf responsive rat MTLn2 mammary adenocarcinoma line. The results of this work have been assembled into a paper, a pre-print of which is submitted as an appendix.

CONCLUSIONS:

1. Transfection of human A375p melanoma or human MDA468 mammary adenocarcinoma cells with the sense gene for human TfR results in transfected cell populations with 1.5 - 4.0 fold more TfR than the vector transfected controls. However, with the A375p/TfR cells, the acquisition of higher TfR expression has no effect on spontaneous or experimental metastatic capability. Continued sorting of higher TfR expressing cell from these populations is underway.

2. Cloning and sorting of human MDA231 mammary adenocarcinoma cells transfected with the cDNA for human TfR results in a population of cells with 6-12 fold more TfR than the vector transfected control population. Metastasis studies using these cells are now underway.

3. Transfection of the human MCF7/LCC2 mammary tumor cell line with the antisense cDNA for human TfR has resulted in cell populations with higher TfR expression than the vector transfected controls. These studies are being re-initated with a new antisense construct.

4. The selection of a highly Tf responsive rat MTLn2 mammary adenocarcinoma cells form a low Tf responsive parental population has resulted in a selected cell line which exhibits greater metastatic capability in syngeneic rats. A pre-print of a paper outlining these studies is included as an appendix.

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The selection of a metastatic rat mammary adenocarcinoma subline from a low metastatic parental population by an *in vitro* process based on cellular ability to proliferate in response to transferrin.

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Abbreviations used: BSA: bovine serum albumin; CAPS: 3-(cyclohexylamino)-1-propanesulfonic acid; CMFH: calcium, magnesium free hank's baisc salt solution; DMEM: Dulbeco's modified eagle medium; FBS: fetal bovine serum; HEPES: N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); αMEM: minimum essential media, alpha modification; PMSF: phenylmethylsulfonyl fluoride; Tf: transferrin; TfR: transferrin receptor; TLCK: N-α-p-tosyl-L-lysine chloromethyl ketone.

Abstract:

We previously found that the proliferative response to transferrin (Tf) and the expression of cell surface transferrin receptors (TfR) correlated with the metastatic capability of various rat 13762NF mammary adenocarcinoma sublines. In order to further assess the involvement of the Tf/TfR system in metastasis, a process was designed and employed whereby those few Tf responsive cells, from the low-Tf responsive, low metastatic 13762NF MTLn2 subline were selected. When maintained in low serum (0.3 % FBS) conditions, no MTLn2 cells plated would survive. However, if the media was supplemented with 0.5 µg/ml rat holo-Tf, a few surviving colonies arose, presumably due to those cell's ability to proliferate in response to the added Tf. These cells were harvested, expanded and exposed to twenty similar rounds of Tf growth selection. The selected cells were designated MTLn2-Tf20. These cells possessed a proliferative response to Tf which was similar to that of the highly metastatic 13762NF sublines. Based on immunofluorescent staining, Scatchard analysis, and on quantitation of affinity isolated TfR, the MTLn2-Tf20 cells possessed 5-6 fold more TfR than did the parental MTLn2 line. When injected into the mammary fat pad of Fisher 344 rats, the MTLn2-Tf20 line produced axillary lymph node metastases in 7/10 animals, and lung metastases in 5/10. No metastases were seen when the MTLn2 parental line was likewise injected. The results indicate that the high Tf-responding cells in a low metastatic tumor population may be more metastatic than the parental population, and the selection of cells with high levels of TfR and proliferative response to Tf produces a subline with greater spontaneous metastatic potential.

Introduction:

Previous studies by us explored the possibility that tumor cell metastasis to a certain target organ was in part enhanced by the ability of the tumor cells to respond to growth factors encountered in the target organ environment. Cells of high and low lung-metastasizing capability from the rat 13762NF mammary adenocarcinoma were examined for their ability to proliferate in response towards media conditioned by lung fragments. A response was seen for the high lung-metastasizing cells only. The major lung-derived lung metastasizing tumor cell mitogen was purified from the conditioned media and was found to be the iron transport protein transferrin (Tf;1-3). Subsequent studies showed a correlation between tumor cell response to transferrin and metastatic capability in 5 of 6 animal and human tumor model systems (4,5).

The transferrin receptor (TfR), a M_r ~180,000 homodimeric integral membrane glycoprotein (6), binds two iron saturated Tf molecules and is responsible for the delivery of iron into cells either through internalization of Fe-Tf (7) or by activation of a plasma membrane associated NADH dependent oxidoreductase which mediates the trans-plasma membrane transport of iron from Tf (8). Rapidly dividing cells, including a variety of tumor cells (6,9,10), usually express high levels of TfR. Other investigators have shown that tumor cell expression of TfR, as determined by histochemical analysis, has been shown to correlate with tumor grade or stage and/or progression and metastasis in human breast carcinomas (11), human bladder transitional cell carcinomas (12), and in human malignant melanoma (13). High levels of TfR have been observed in a metastatic lesion of a maxillary neoplasm, but not in the parental tumor (14). Transferrin receptor expression was seen to be increased in a human melanoma line selected for metastatic capability in nude mice, when compared to the poorly metastatic parental population (15). Transferrin has been shown to be the major mitogen in bone marrow for bone marrow metastasizing human prostatic carcinoma cells (16), and proliferative response to Tf has been shown to be associated with malignant progression in a series of murine B16 melanoma sublines (17).

Transferrin's proliferative effect on cells is thought to be due primarily to its ability to transport iron into the cell, thus maintaining the activity of key enzymes required for proliferation. One major site of iron need in rapidly dividing cells is the enzyme ribonucleotide reductase (6) . However, some studies have suggested that iron transport alone cannot explain transferrin's growth stimulating activity (18-20). Also, iron

delivered by Tf has been shown to mediate processes that soluble iron can not (21), and stimulation of the TfR through transferrin-independent means has been shown to induce a number of activities in T cells (22).

The objective of this study was to modulate tumor cell response to transferrin using a selection technique which would allow only the survival of those rat mammary adenocarcinoma cells which possessed the ability to respond to transferrin, and to determine whether this selected population's metastatic capability was different than that of the low Tf-responding, low metastatic parental line.

Methods:

Cells and Cell Culture:

The MTLn2 line was originally cloned from a lung metastasis arising from a 13762NF mammary fad pad tumor. However, this line displayed little or no metastatic ability upon re-injection into syngeneic Fisher 344 rats (23). The line was maintained at 37°C in a humidified 5% CO_2 atmosphere in α-MEM containing 5% (v/v) fetal bovine serum (FBS). The line was routinely tested for mycoplasma.

Rat holo-transferrin preparation:

Rat apo-transferrin (Sigma, St. Louis, MO) was dissolved at 1 mg/ml in 25 mM sodium bicarbonate, pH 8.0 containing 1 mg/ml ferric ammonium citrate. After a 1 hour incubation at 25°C, the solution was dialyzed extensively (2 X 4L) against 25 mM HEPES, pH 7.5.

Selection Procedure:

Cells were removed from stock plates with 0.25% trypsin, 2 mM EDTA (in Ca^{++}, Mg^{++} free PBS) and seeded onto 100 mm plates at a density of 2,000 cells/plate in 10 ml α-MEM containing 1% FBS. One day later, media was changed to 10 ml α-MEM containing 0.3% FBS and iron saturated rat transferrin was added to selection plates to a final concentration of 0.5 μg/ml. Control plates received the corresponding amount of transferrin solvent (25 mM HEPES buffered DMEM [pH 7.5] containing 10 mg/ml BSA) only. Seven days later, cells were removed from the selection plates with 0.25% trypsin, 2 mM EDTA, and re-seeded onto 100 mm cell culture plates at 2,000 cells/plate. The selection process was then repeated for each round. For selection rounds 11-20, FBS was eliminated from the media.

Cell Growth Assays:

Cells were removed from stock plates and seeded in 100 µL α-MEM containing 1% FBS at a density of 2,000 cells/well in 96 well plates. One day later, media was changed to 100 µL α-MEM containing 0.3% FBS, and increasing amounts of holo-rat transferrin was added into test wells. Five days later, cells were quantitated using a crystal violet stain assay: cells were washed with PBS, and were fixed at 25°C for 30 m with PBS containing 5% v/v glutaraldehyde. Fixed cells were washed with water, allowed to dry., and were stained for 30 min. at 25 °C with 50 µL 0.1% crystal violet in 50 mM CAPS, pH 9.5. Stained cells were washed with water, and were solubilized with 50 µL 10% acetic acid. Cell number was determined by reading Absorbance at 590 nm on a Dynatech MR5000 plate reader. Absorbance in this system correlates with cell number up to 40,000 - 50,000 cells/well (2,24).

Immunofluorescent Detection of Cell Surface Transferrin Receptors.

Cells grown on LabTek (Nunc, Naperville, IL) slides were washed 3 times with PBS and equilibrated to 4° C. The primary antibody (anti rat transferrin receptor: OX-26; BPS, Indianapolis, IN) or normal mouse IgG were diluted at 1:100 in 4°C PBS containing 10 mg/ml BSA . The antibody or IgG solution was added to the cells and the slides were incubated on ice for 2h. The cells were washed 3 times with PBS-BSA and were incubated on ice with a 1:100 dilution (in PBS-BSA) of an Fab' fragment of phycoerythrin conjugated rabbit anti-mouse IgG (Zymed, San Francisco, CA) . The cells were washed 3 times with PBS-BSA and were examined for fluorescence using a Nikon Diaphot phase contrast microscope.

FACS analyses:

All washes and antibody incubations were performed using 25mM HEPES (pH 7.5) buffered Dulbeco's modified minimal essential media containing 10mg/ml BSA (DMEM-BSA). Cells were removed from culture plates using 0.25% trypsin, 2 mM EDTA (in Ca^{++}, Mg^{++} free PBS). As soon as detachment was complete, FBS was added to 1% (v/v), to neutralize the trypsin. Cells were washed 2X by centrifugation and resuspension in DMEM-BSA. Cells were counted, cell density was adjusted to 1X 10^6/ml, and the cell suspensions were equilibrated to 4°C. Normal mouse IgG or anti-rat-TfR (final dilution = 1:100) was added to the suspensions. Cells were incubated at 4°C for 1h, washed 2X with DMEM-BSA and were incubated at 4°C with a 1:100 dilution of an Fab' fragment of phycoerythrin conjugated rabbit anti-mouse IgG. Cells were washed 2X with DMEM-BSA and were analyzed for fluorescence using a Becton-Dickinson FACStar instrument.

Spontaneous Metastasis Assays:

Cultured cells to be injected were removed from plates with calcium, magnesium free Hank's basic salt solution (CMFH) containing 1 mM EDTA and 0.25 % trypsin. Cells were washed 3 X with CMFH, cell density was determined using a model ZM Coulter Counter and the density was adjusted to 5 X 10^6/ml with CMFH. Cells were kept at room temperature and were injected in a 0.2 ml volume into the left mammary fat pad (2 cm anterior to the hind leg) of Metofane (Pittman-Moore) anesthetized syngeneic female Fisher 344 rats (age: 6 - 8 weeks). Six weeks later, animals were sacrificed with an overdose of Metofane and were examined for the presence of metastatic lesions.

Affinity Isolation of Transferrin Receptors using Immobilized Transferrin:

Based on published procedures (25,26). Cyanogen bromide activated agarose was washed with 10 volumes of 1 mM HCl and equilibrated in a 0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3 coupling buffer. Apo-transferrin was added to the agarose in coupling buffer at a ratio of 10 mg protein /ml packed gel. The mixture was incubated at 4° C overnight in an end over end mixer. The gel was treated with 1 M ethanolamine, pH 8.0 for 2 h at 25° C and was washed with coupling buffer, then with 0.1 M acetate, pH 4.0, containing 0.5 M NaCl, and again with coupling buffer. The immobilized Tf was iron saturated by exposure to 1 mg/ml ferric ammonium citrate in 0.1 M NH_4HCO_3. The gel was washed with PBS containing 0.5% v/v Triton X-100 (TX100).

To reduce inherent bound Tf, cells were incubated in two changes of α-MEM only (two h each) prior to the analysis. Cells (70 -80 % confluent, in 100 mm dishes) were then washed with 3 X 5 ml PBS at 4°C, and 3 ml 4°C PBS containing 1.0 mg NHS-LC-biotin was added. Dishes were incubated at 4°C for 90 min., while shaking. Cells were washed 5 times with 3 X 5 ml PBS and lysed in 3 ml (per dish) PBS containing 2.0% Triton X-100, 0.1 mM PMSF, 0.1 mM TLCK, 0.1 mM iodoacetamide, 10 µg/ml leupeptin, and 10 µg/ml aprotinin, at 4°C for 2h. The lysate was centrifuged at 13,000 X g for 10 min. Lysate supernatant protein was determined using the detergent compatible BCA (Pierce, Rockford, IL) assay.

The lysate supernatant (1 - 2 mg total protein) was combined with an excess of Tf-agarose (0.4 ml packed gel) and incubated for 2 h at 37°C. The gel was harvested by centrifugation at 2,000 x g, and was washed 3 times by suspension in PBS containing 2% Triton X-100, and repeated centrifugation. Bound cell lysate proteins were released

by exposure of the gel to 0.4 ml non-reducing SDS-PAGE sample treatment solution at 95°C for 10 minutes. The samples were separated on a 7.5 % SDS-PAGE gel run according to Laemmli (28) and blotted onto an immobilon (Millipore, Bedford, MA) membrane using a 10 mM CAPS, pH 10.0 transfer buffer. The membrane was blocked for 2 h at 25 °C with PBS containing 10% nonfat dry milk and 0.5 % Tween 20. The membrane was incubated with block solution containing a 1:2000 dilution of streptavidin-HRP (Boehringer Mannheim,) for 1h at 25°C. The membrane was washed 4X with 40 ml PBS containing 0.5% Tween 20. HRP-ECL substrate (DuPont, Wilmington, DE) was applied and light emitting bands were detected by autoradiography. Bands were quantitated using a Hoeffer (San Francisco, CA) model GS300 scanning densitometer.

Scatchard analysis of cell surface TfR numbers:

Iodination of Tf: to 1 ml of a 1 mg/ml solution of Rat holo-Tf (in 25 mM HEPES, pH 7.5) was added 0.5 mCi of $Na^{125}I$ (ICN, Cleveland, OH) and 10 υL of a 10 mg/ml solution of chloramine-T. This mixture was incubated at 25°C for 1 h and 10 μL of a 20 mg/ml of sodium bisulfite was added. The sample was passed through a PD-10 column (Pharmacia, Uppsala, Sweden) equilibrated and run with PBS. One ml fractions were collected and aliquots of each fraction were counted on a gamma counter. Radioactive fractions corresponding to the void volume were pooled. Specific counts were ≈ 300,000 cpm/μg protein

Cells were grown in 12 well plates to 70-80% confluency. To reduce inherent bound Tf, cells were incubated in two changes of α-MEM only (two h each) prior to the analysis. Cells were washed twice with PBS, and 1 ml PBS containing 1% v/v liquid gelatin (Sigma, as a non-specific blocking agent) was added. The plates were equilibrated to 4 °C and increasing levels of ^{125}I-Rat Tf (from 0.33 to 6.0 μg/well)was added to wells (in quintuplicate). Immediately prior to the addition of ^{125}I-Rat Tf, two wells of each dose received a 500 fold excess of cold Tf. Wells were incubated for 2h at 4°C and were washed 5X with PBS. Cells were lysed with PBS containing 2% Triton X-100, lysate were placed into scintillation vials, and cell bound counts were determined using a Packard A5550 gamma counter.

Results

When low metastatic rat MTLn2 mammary adenocarcinoma cells were plated at 2,000 cells/10 cm dish and grown in α-MEM containing only 0.3% FBS, none of the cells originally seeded would survive. However, if the same culture was supplemented with 0.5 μg/ml rat transferrin, 2 - 10 colonies of surviving cells would arise from the 2,000 initially plated. When these cells were harvested and re-exposed to the same transferrin selection conditions, 10-30 colonies would appear, and as rounds of selection continued, increasing numbers of surviving cells would become apparent. After 18-20 rounds of similar selection procedures, the selected population would produce a near confluent plate. Furthermore, during the last ten rounds of selection of transferrin responsive cells, FBS could be eliminated from the system, resulting in greater selection pressures. With each round of selection, cells always exhibited no ability to survive in Tf free conditions. After twenty rounds of selection, an MTln2 population was obtained (MTLn2-Tf20) which demonstrated a marked enhanced ability to proliferate in response to Tf than did the parental MTLn2 cells (Figure 1). Either line grew at the same rate in conventional culture conditions: α-MEM supplemented with 5% FBS (Figure 2).

It was of interest, then, to determine if cell surface TfR was increased in the MTLn2-Tf20 cells. Immunofluorescent microscopy analysis of cell surface TfR revealed visibly greater cell surface fluorescence in the selected population when compared to the parental cells (Figure 3). Analysis of cell surface TfR expression by FACS was also performed. Cells were rapidly removed from plates with trypsin, stained for TfR at 4°C, and analyzed for fluorescence using a Becton-Dickinson FACstar instrument. This analysis indicated that there was 12 fold more TfR on the MTLn2-Tf20 line than on the MTLn2 line (Figure 4). Cell surface TfR could be biotinylated and methods to quantitated TfR were developed to take advantage of this. As the anti-rat TfR antibody used for immunofluorescent studies (OX-26) was unable to immunoprecipitate biotinylated TfR (data not shown), a procedure based on affinity isolation of TfR (25,26) was employed. Cells were surface biotinylated, lysed with PBS containing Triton X-100, and the resulting solubilized cell material was exposed to immobilized Tf. The agarose-Tf preferentially bound TfR in the lysate, which was released with SDS-PAGE sample treatment solution. The released TfR could be measured by SDS-PAGE followed by blotting, whereby detection of biotinylated bands could be accomplished by incubation of the blot with streptavidin-HRP followed by ECL using an HRP substrate. This method

indicated that the MTLn2-Tf20 possessed 5-6 fold more TfR on a per cell basis than did the Parental MTln2 cells (Figure 5).

To verify the TfR affinity isolation results and to assess any changes in the two lines affinity for Tf, conventional Scatchard analysis using ^{125}I-Tf was performed. Liquid gelatin was found to be an ideal blocking agent for this, as non-specific binding obtained with it was reduced to near background. In agreement with other assays, Scatchard analysis revealed that the MTLn2-Tf20 possessed 5-6 fold more surface TfR than did the MTln2 cells. No significant differences in affinity for Tf were seen (Figure 6).

To ascertain the full metastatic behavior of both lines, spontaneous metastasis assays were performed. When 1 x 10^6 cells of either line were injected into the left mammary fat pad of syngeneic Fisher 344 rats, primary tumors formed at equal (70-80%) frequency and grew to a similar range of sizes. After six weeks of tumor growth, 7/10 rats receiving MTLn2-Tf20 displayed palpable left axillary lymph node metastases (Table 1). Upon sacrifice, necropsy revealed that 5/9 MTLn2-Tf20 receiving rats had lung metastases . No metastases were evident in any of the animals injected with the parental MTLn2 line (Table 1). One of the rats receiving MTLn2 cells expired one week after injection and was dropped from the group. Of note in these *in vivo* studies was that the MTLn2-Tf20 primary tumors displayed a hollowed out necrotic center whereas the MTLn2 primary tumors were solid.

An intermediary *in vivo* experiment was performed comparing the spontaneous metastatic capability of MTLn2 and MTLn2-Tf10 cells. The procedure followed was identical to that for the MTLn2/ MTLn2-Tf20 experiment, except that six rats were used in each group. In that study, the MTLn2-Tf10 cells formed lung metastases in 1/6 rats and left axillary lymph node metastases in 3/6 rats. The only metastasis seen with the MTLn2 cells was an axillary lymph node metastasis in one animal. It appeared as though the metastatic capability of the MTLn2-Tf10 line was in a range between the MTLn2 and MTLn2-Tf20 lines. The proliferative response of MTLn2-Tf10 cells to Tf was also intermediate in magnitude when compared to the MTLn2 and MTLn2-Tf20 cells (data not shown). Cell surface TfR in this line was not measured.

Discussion:

Previous results generated by us (1-5) and findings from other laboratories (11-17) have indicated that in certain cell systems, tumor cell proliferative response to Tf and/or expression of TfR correlates with metastatic capability.

The observation of a cellular property associated with and perhaps thought to be responsible for metastatic activity has frequently led metastasis researchers to pursue *in vitro* selection techniques whereby tumor cells possessing high or low levels of a metastasis-associated marker or activity could be isolated. The proposed influence on metastatic behavior that the selected property played could then be determined by assessing the metastatic capability of the selected cells *in vivo* (28-31). These types of experiments have resulted in the identification of a number of tumor cell phenotypes thought to be associated with the ability to form metastases (28-31)..

Therefore, our results naturally raised the question as to whether or not tumor cell metastatic behavior could be altered by artificially manipulating the Tf/TfR system. A number of processes were considered where Tf responsiveness and/or TfR expression could be enhanced in a low metastatic line (to hopefully create a high metastatic line) or where those same characteristics could be reduced in a high metastatic line, to perhaps decrease its metastatic capability. We had previously performed Tf growth response assays (1,2), where highly lung metastatic rat mammary adenocarcinoma cells were seen to proliferate in response to Tf in low serum conditions, whereas low metastatic cells proliferated to a much less degree, or not at all. A logical method to select Tf responsive cells from a low metastatic population then, was to scale-up these Tf growth assays into an *in vitro* selection process whereby those few cells from the low metastatic population that could respond to Tf could be isolated, expanded, and re-selected.

The low metastatic MTLn2 line from the rat 13762NF mammary adenocarcinoma series was the ideal choice for these studies since its growth rate in usual culture conditions is equal to the more highly metastatic 13762NF MTLn3 line (23). Also, the MTLn2 line is totally unable to survive in low or no serum conditions. Other low metastatic lines from the 13762NF series (i.e.: MTPa) were less well suited to the selection process since their basal growth rate is much less than that of the highly metastatic lines (23), and they demonstrated greater survival properties in low serum conditions. This last characteristic contributed background non-Tf responsive cells to the selected population.

Finally, the 13762NF series was an ideal choice for this experiment since it is a syngeneic tumor system and the various sublines generated from it were originally selected based on their differences in spontaneous metastatic capability (23). Also, the metastatic spread of cells in the 13762NF series mimics the metastasis of mammary adenocarcinoma cells in humans, with an initial metastatic migration to the lymph node(s) followed by metastasis to other organs.

Results with the MTLn2 line were obtained relatively rapidly because of those cell's rapid replicative capability and their ability to form large primary tumors in a comparatively short time. Other animal tumor cell lines of a low metastatic nature, from other tumor systems are currently being selected for high Tf responding cells in this same manner, however those selections are currently in their early stages.

Other strategies are also being tested for their ability to manipulate tumor cell TfR numbers, and hopefully, Tf responsiveness. This is being approached primarily using transfection techniques, where plasmid constructs encoding sense and antisense cDNA for the human TfR are being transfected into low and high metastatic cells, respectively.

A subset of low metastatic rat MTLn2 mammary adenocarcinoma cells which possess high TfR numbers and the ability to proliferate in response to Tf was selected from the low TfR expressing, low Tf-responsive parental population. The selected cells demonstrated a much greater ability to spontaneously form axillary lymph node and lung metastasis in syngeneic rats than did the parental line. The results confirm previous observations and indicate that tumor cell ability to respond to Tf can be an important characteristic for metastasis formation, in some tumor cell systems. The results indicate that continued exploration into therapeutic strategies which interfere with tumor cell Tf binding or iron uptake (32,33) are warranted.

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Figure Legends:

Figure 1: Proliferative response of MTLn2 and MTLn2-Tf20 cells to transferrin. Cells were plated in α-MEM containing 1% FBS .at a density of 2,000 cells /well in 96 well plates. One day later, media was changed to that containing 0.3% FBS and increasing amounts of rat holo-Tf was added. Five days later, cell numbers were determined using a crystal violet stain procedure . Points represent the mean ± SD of four wells.

Figure 2: Growth rate of MTLn2 and MTLn2-TF20 cells in stock culture conditions. Cells were plated in α-MEM containing 5% FBS .at a density of 2,000 cells /well in 96 well plates. Five days later, cell numbers were determined using a crystal violet stain procedure . Points represent the mean ± SD of four wells.

Figure 3: Immunofluorescent analysis of cell surface TfR on MTLn2 and MTLn2-Tf20 cells. Cells were grown on LAB-TEK chamber slides and were stained at 4°C with the OX-26 anti-rat transferrin antibody followed by a phycoerythrin conjugated Fab' fragment of anti-mouse IgG. Cells were then examined for fluorescence using a Nikon Diaphot microscope. A: MTLn2, B:MTLn2-Tf20. Cells were at approximately equal density. Cells initially treated with an irrelevant mouse IgG produced no fluorescence (data not shown).

Figure 4: Analysis of cell surface TfR using FACS. Cells were removed from culture plates with trypsin/EDTA, were stained at 4°C with the OX-26 anti-rat TfR antibody followed by a PE conjugated Fab' fragment of anti-mouse IgG, and were analyzed for fluorescence using a BD FACstar instrument. The median fluorescence for the MTLn2 line was 36.2, and for the MTLn2 Tf20 line was 445.1. When either cell line was initially incubated with normal mouse IgG1, a mean fluorescence of 4.0-5.0 was seen (data not shown).

Figure 5. Results of cell surface TfR measurement in MTLn2 and MTLn2-Tf20 cells by affinity isolation of biotinylated TfR. Cells were biotinylated at 4°C, lysed, and equal cells equivalents of cell lysates were exposed to an excess of Tf-agarose. The agarose was collected, washed, and bound lysate proteins were separated by SDS-PAGE. Following Western blotting of bound proteins, biotin groups were detected by exposure of the blot to streptavidin-HRP and application of an ECL HRP substrate.

Figure 6. Scatchard analysis of Tf binding to MTLn2 and MTLn2-Tf20 cells. Cells were grown to 70-80 % confluency on 12 well plates, were washed 2X with serum free media, and were equilibrated to 4°C. Increasing amounts of ^{125}I-TF was added to wells in quintuplicate. 2h later, wells were washed, cells were lysed, and cell bound (lysate) radioactivity was determined using a gamma counter. Non-specific binding wells received a 200 fold excess of cold Tf in addition to the ^{125}I-TF. Non-specific counts were subtracted form all points prior to graphing.

Figure 7: Photographs of representative lungs from rats injected with the MTLn2 parental line (above) or the MTLn2 Tf20 line (below). The lungs from the animals injected with MTLn2 Tf20 exhibiting the greatest number of lung metastases are shown.

Table 1. Results of spontaneous metastasis assays. Syngeneic female Fisher 344 rats were injected with 1 X 10^6 MTLn2 or MTLn2 Tf20 cells into the left mammary fat pad. Six weeks later, rats were sacrificed and examined for gross inguinal, axillary, and lung metastasis.

Figure 1.

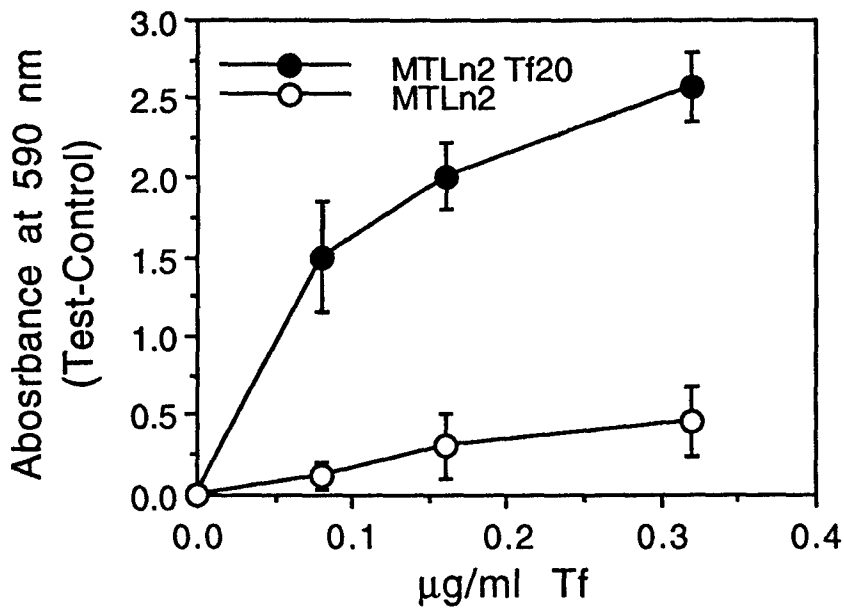
Absorbance at 590 nm
(Test-Control)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
MTLn2 Tf20
MTLn2
0.0
0.1
0.2
0.3
0.4
µg/ml Tf

Figure 2.

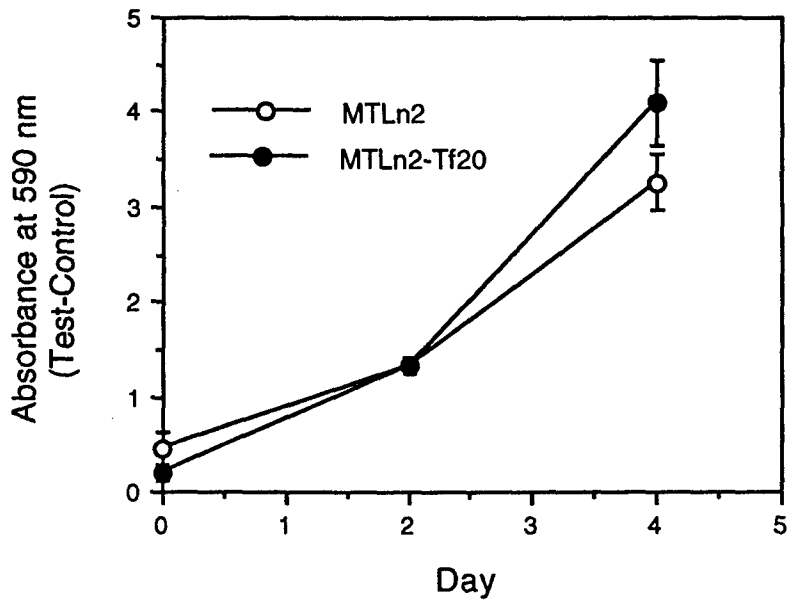
Absorbance at 590 nm
(Test-Control)
MTLn2
MTLn2-Tf20
0
1
2
3
4
5
Day

Figure 3.

A: **B:**

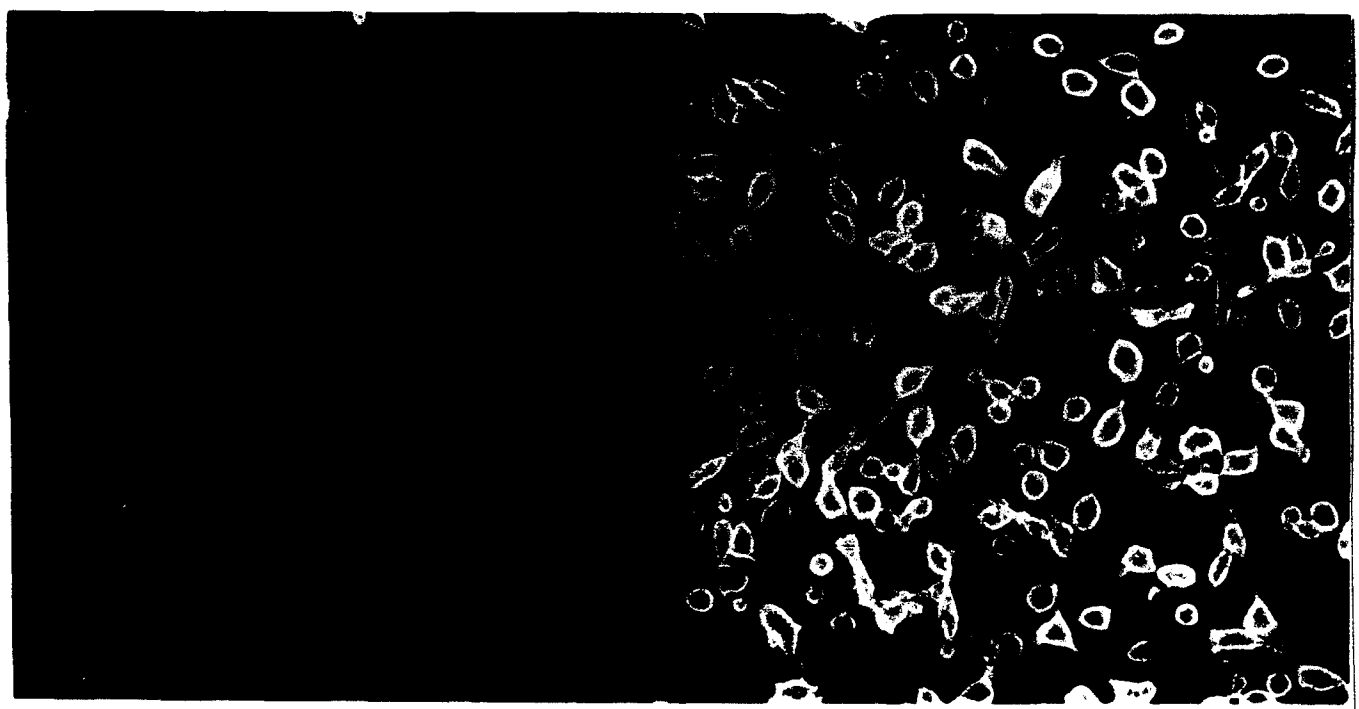

Figure 4.

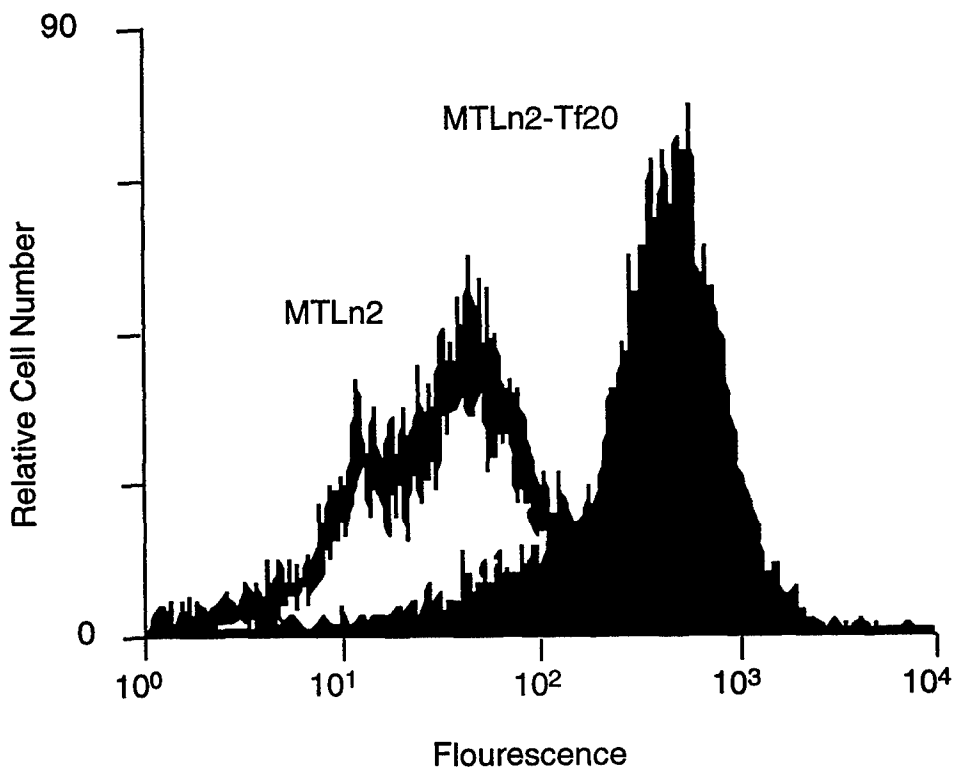
90
0
Relative Cell Number
MTLn2-Tf20
MTLn2
10^0
10^1
10^2
10^3
10^4
Flourescence

Figure 5.

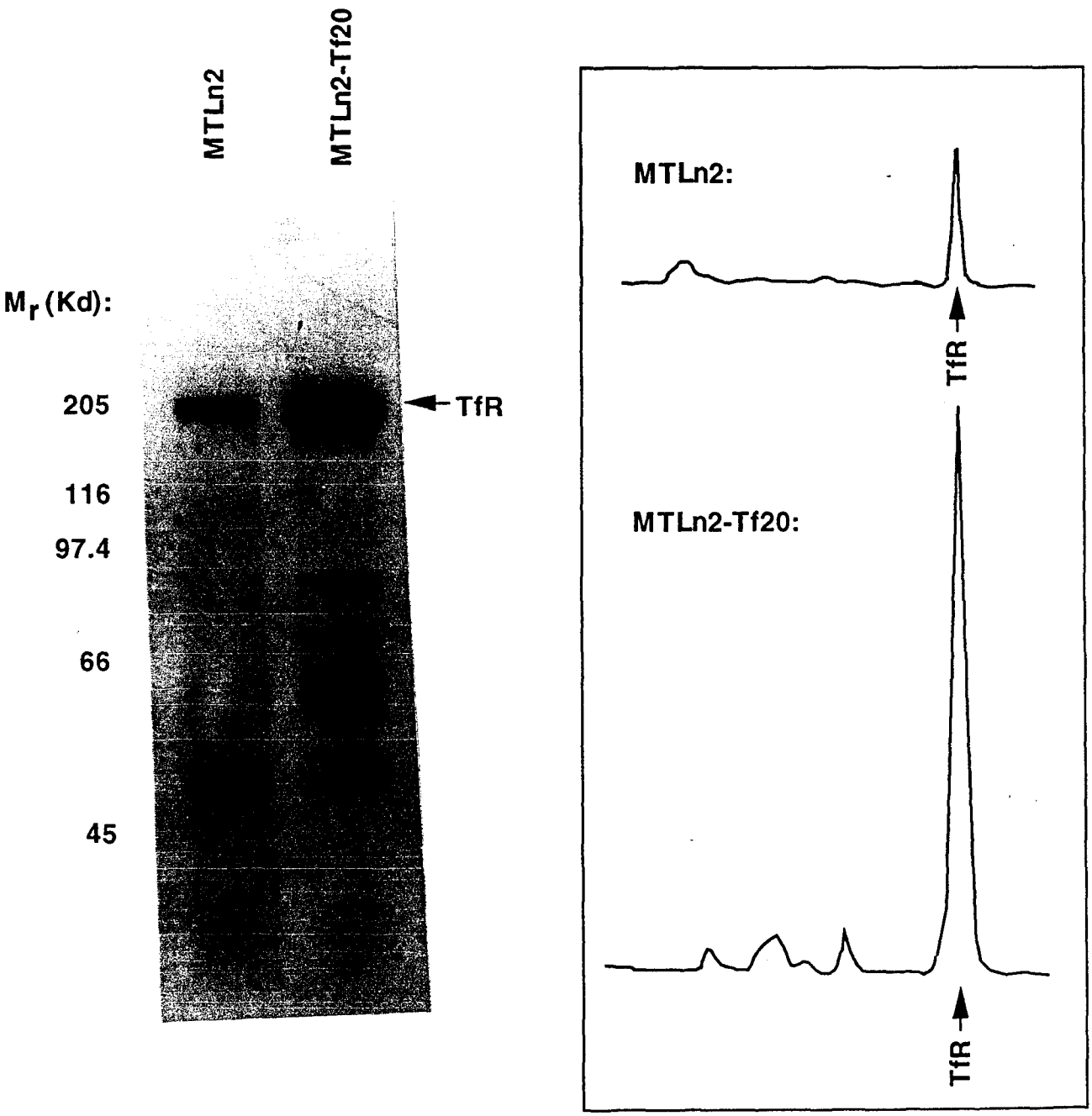
MTLn2
MTLn2-Tf20
M_r (Kd):
205
116
97.4
66
45
TfR
MTLn2:
TfR
MTLn2-Tf20:
TfR

Figure 6.

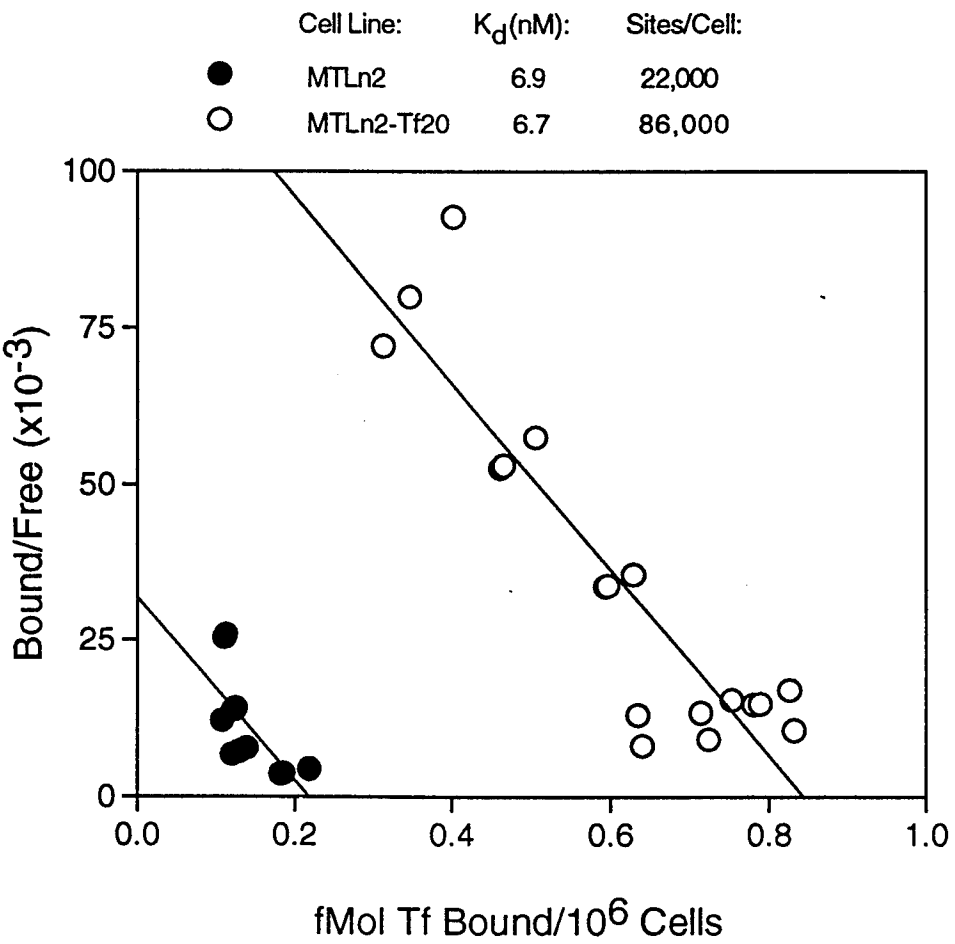
Cell Line:
K_d(nM):
Sites/Cell:
MTLn2
6.9
22,000
MTLn2-Tf20
6.7
86,000
100
75
50
25
0
Bound/Free (x10^{-3})
0.0
0.2
0.4
0.6
0.8
1.0
fMol Tf Bound/10^6 Cells

Figure 7.

MTLn2:

MTLn2 Tf20:

Table 1: Spontaneous Metastasis Assays:

MTLn2 Tf20:

Rat Number:	1° Tumor Average Diameter (mm)	Axillary Lymph Node Tumor Average Diameter (mm)	# of Lung metastases
1	28	8	>50
2	18	7	>50
3	20	8	28
4	22	18	0
5	13	0	0
6	0	0	0
7	5	0	8
8	15	6	>50
9	18	4	>50
10	8	0	0

MTLn2:

Rat Number:	1° Tumor Average Diameter (mm)	Axillary Lymph Node Tumor Average Diameter (mm)	# of Lung metastases
1	20	0	0
2	25	0	0
3	13	0	0
4	0	0	0
5	4	0	0
6	0	0	0
7	17	0	0
8	32	0	0
9	4	0	0

Table 1. Results of metastasis assays. Syngeneic female Fisher 344 rats were injected with 1 X 10^6 MTLn2 or MTLn2 Tf20 cells into the left mammary fat pad. Six weeks later, rats were sacraficed and examined for gross inguinal, axillary, and lung metastasis.